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# The Anaerobic Actinomyces in Endodontics

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# THE ANAEROBIC ACTINOMYCES IN ENDODONTICS

by

Orlando Muñoz-Noya, DDS

A Thesis Submitted to the Faculty of the Graduate School  
of Loyola University in Partial Fulfillment of  
the Requirements for the Degree of  
Master of Science

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Dr. Marshall Smulson, chairman of the Department of Endodontics at Loyola University School of Dentistry.

Dr. John Madonia, chairman of the Department of Microbiology at Loyola University School of Dentistry.

## BIOGRAPHY

Orlando Muñoz-Noya was born in Humaco, Puerto Rico the 19th of May, 1932. He attended elementary school in Manati, Puerto Rico and graduated from Manati High School in the year 1949. After college at the University of Richmond, Virginia, he entered Dental School at Loyola University of the South, New Orleans, Louisiana, from where he obtained his DD S in 1956. He entered the United States Army Dental Corps that year and remained with the Army until 1960. He practiced general dentistry for six years, and re-entered the U.S. Army in 1966. In 1968 he was selected to attend Loyola University School of Dentistry, Chicago, Illinois, to obtain further education in Endodontics.

Major Muñoz was married in 1956 to Diana Vanessa Montes, and at this time they are the parents of five children. He intends to remain with the U.S. Army until he is eligible for retirement.

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## CHAPTER I

## INTRODUCTION

When using routine root canal culturing techniques, a small percentage of cases undergoing endodontic treatment will yield negative cultures, yet appear to have symptoms which indicate persistent infection. It is logical to suspect that the infecting microorganism has not been detected during the sampling procedure. Since root canals of teeth provide an environment conducive to growth on anaerobic bacteria, the prime suspect which has defied detection can be an anaerobic organism. Among the anaerobes inhabiting the normal human oral cavity is Actinomyces israelii. This organism has been found to be the principal causative entity of human actinomycosis. The method by which this microbe is able to penetrate the tissues and cause disease is still a mystery. Actinomyces israelii has been recovered from root canals of patients not suffering from actinomycosis by some investigators of the flora of infected root canals. Invasion through the root canal and into the periapical tissues by this organism is not far fetched. The object of this study is to compare different culture media commonly used in the practice of endodontics as to their ability to detect the presence of Actinomyces israelii and sustain its growth, and also to determine the relative number of such organisms required to initiate detectable growth on the different media tested.

## CHAPTER II

### REVIEW OF LITERATURE

#### I. Culture Media in Endodontics

Since microorganisms may play an important part in the pathogenesis of pupal and periapical disease, one of the most important objectives in endodontic practice is to eliminate them. At present the only available means of determining whether this has been attained is bacteriologic sampling of the contents of the root canal. Assuming that an acceptable, strict regimen of bacteriologic control during the technical aspect of obtaining the culture is observed, the accuracy of the procedure depends then, on the ability of the culture medium to support the growth of the microorganisms which may be found in the root canal.

The ideal culture medium for endodontic use should support the growth of all aerobic, anaerobic, and microaerophilic organisms as well as inactivate the in vitro bacteriostatic effect of commonly used chemotherapeutic agents. Meat or vegetable infusion broth with appropriately added enrichments such as 0.1% to 0.2% dextrose, soluble starch or yeast extract, 5% to 10% serum, whole blood or ascitic fluid, and 0.1% to 0.2% agar are in everyday use. Very popular among endodontists are brain heart infusion broth, trypticase soy broth, thioglycollate broth, and glucose ascites broth.

Morse and Yates in 1942 tested sodium thioglycollate as a possible culture medium to detect the presence of anaerobes in root canals. They found that sodium thioglycollate was not efficient for the cultivation of anaerobic organisms which may be found in root canals. In 1947, Shay compared several culture media as to their ability to support growth of microorganisms isolated from root canals of 184 infected teeth. At the same time, he attempted to find a suitable culture medium for growth of anaerobic bacteria. In this study, Shay tested trypticase dextrose (pH 7.2), brain heart infusion (pH 7.4), serum dextrose (pH 7.4), brain agar (pH 7.4), Brewer's thioglycollate (pH 7.4), and trypticase dextrose (pH 5.5). Best results were obtained with trypticase dextrose (pH 7.2) and brain heart infusion broth (pH 7.4). Using these two culture media, a 97.8% positive result was obtained from the 184 known infected root canals and he recommended that both media be used for bacteriologic sampling of root canals. Another conclusion reached by this study was that strict anaerobic conditions were not necessary for the cultivation of anaerobic organisms from the root canals.

Leavitt, Naidorf, and Shugaevsky, in 1955 conducted a study of the possible role anaerobic organisms played in endodontic infections. Various observations made by endodontists led to the suspicion that anaerobic bacteria remained infecting root canals of teeth not responding to proper therapy even though negative cultures were obtained with culture media then commonly



used. A closed system using alkaline pyrogallol as an oxygen absorber was used in the incubation of anaerobic microorganisms. This technique was subsequently modified using sodium hydrosulfite as a reducing agent which permitted the cultivation of organisms under anaerobic conditions on a single test tube basis. Dextrose broth was used as a standard medium in the comparison of the two systems. Of thirty-five cases which had yielded two successive negative cultures using aerobic cultivation, five cases, or 14.3% of the total showed growth when tested under anaerobic culture procedures.

In a follow-up study, Leavitt, Naidorf, and Shugaevsky, in 1955, investigated the possibility of having one culture medium which could support both the growth of aerobes and anaerobes in the same tube. They discovered that the addition of small amounts of agar to a broth would provide varying degrees of anaerobiosis. The inclusion of agar prevents the oxygen absorbed at the surface from diffusion through to the bottom of the tube and provides a column of culture medium with decreasing oxygen tension. Their study also showed that precautions which enhanced the anaerobic environment were:

- (1) Use of screw capped culture tubes instead of cotton plugged ones, since the cotton allowed for gradual oxygenation of the medium.
- (2) There must exist a favorable ratio of diameter to length of tube. This was found to be 16 mm. x 125 mm.

- (3) The culture tubes were not to be shaken since agitation would enhance oxygenation of the lower strata of the medium.

Dextrose broth, thioglycollate broth, and trypticase soy broth were prepared with and without the addition of 0.1% agar. The test organism for the aerobic conditions was Staphylococcus aureus and the anaerobic test organism was Clostridium histolyticum. The medium which showed most sensitivity in supporting the growth of both the aerobes and anaerobes was trypticase soy broth with 0.1% agar. This medium was also fluid enough to allow the paper point which was used to obtain the sample to settle at the bottom of the tube. The strongly aerobic organisms grew only at the top third of the culture tube and the anaerobic organisms were confined to the bottom of the tube. Brewer's thioglycollate without agar was unable to support significant anaerobic growth from the small inoculum used.

A two-year clinical test of trypticase soy broth with 0.1% agar by Leavitt and co-workers (1958) showed it to be sensitive to anaerobic as well as aerobic bacteria. The percentage of anaerobic microorganisms recovered on this medium from infected root canals was comparable to that shown by using strict anaerobic laboratory procedures. The medium also was found to allow the growth of most aerobic organisms found in infected root canals. Cobe, Chilton, and Kaufman (1957) studied several culture media with

varying concentrations of agar as to their ability to support the growth of both aerobic and anaerobic bacteria. The culture media tested were Brewer's thioglycollate broth, trypticase soy broth, brain heart infusion broth, and 1% dextrose broth. Agar in concentrations of 0.1%, 0.15%, 0.2%, 0.3%, and 0.5% was added. They found that trypticase soy with 0.2% agar was most suitable for the cultivation of both anaerobes and aerobes, and they also found this medium very adequate for the cultivation of yeast forms. Detection of anaerobes was better with this medium than with others tested. The addition of agar in concentrations over 0.2% would create media which would be too solid to allow for penetration of the sampling absorbent paper point

Van Amerongen, Slaterus, and Eggink (1957) compared sheep blood bouillion, Levinthal's agar, Brewer's thioglycollate broth with 0.05% agar, and trypticase agar dextrose broth against brain heart infusion broth. Cultures were taken in screw capped bottles and also in cotton plugged tubes. They found Levinthal's agar to be inferior, while the quality of the other culture media appeared to be equal. Brewer's thioglycollate broth, often recommended for detection of anaerobic microorganisms did not prove in their study to be superior to the other culture media. They also found no difference between results obtained using cotton plugged tubes and screw capped tubes.

Ah Moo in 1964 compared the sensitivity of trypticase soy broth with 0.1% agar, thioglycollate medium (135°C)\* and brain heart infusion broth. The cultures were incubated at 33°-37°C and evaluated at 24 and 48 hour intervals. Where no growth was noticed at the 48 hour interval, the cultures were incubated an additional twelve days. He found that there was a difference in sensitivity in the three media tested. Trypticase soy broth with 0.1% agar was the most sensitive for all time intervals.

Nolte reported in his book Oral Microbiology (1968), that the addition of agar to media favored the development of isolated colonies throughout the medium in the individual tube. He reported that trypticase soy broth with agar was most sensitive and as far as agar concentrations, he favored 0.2% because this allowed for better separation of colonies throughout the tubed media than 0.1%. Ingle (1967) has recommended the use of trypticase soy with 0.2% agar with pH 7.2.

Grossman, in 1966, reported on the minimum number of oral microorganisms needed to initiate growth in several culture media. The media tested were trypticase soy broth with 0.1% agar, thioglycollate broth, and cooked meat medium. The culture media were inoculated with serial dilutions of Streptococcus salivarius, Streptococcus mitis, Streptococcus faecalis, and Staphylococcus aureus. He found that brain heart infusion

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\*BBL - Baltimore Biological Laboratories, Cockeysville, Maryland

broth with 0.1% agar which was later included in the study and trypticase soy broth with 0.1% agar were the most sensitive and would support the growth of bacteria even though the inoculum consisted of a small number of microorganisms. For this particular test he found no superiority of one culture medium over the other, but he recommended the use of brain heart infusion broth with 0.1% agar, and the addition of 5% ascitic fluid or 10% horse serum for the growth of the more fastidious organisms.

Sommer, Ostrander, and Crowley (1966) advocated the use of glucose ascites medium. The main constituent of this medium is beef infusion broth or brain heart infusion broth to which small amounts of glucose and agar are added. Five to ten per cent sterile ascitic fluid is added after autoclaving of the medium and cooling to 45° F. The final pH should be 7.4. Glucose enhances the growth of staphylococci, lactobacilli, and yeasts.

Later studies by those investigating endodontic culture media were directed toward the possibility of transfer of intracanal medication into the culture tubes, thus inhibiting growth of any microorganism which might have existed in the root canal. This interest came about due to the introduction of a wide range of chemotherapeutic agents and antibiotic preparations in the practice of endodontics. Thus it was decided that inactivators should be added to culture media so as to neutralize the in vitro antibacterial effect of the medicaments used and thereby permit the infecting microorganisms to grow.

Recently it has been suggested that sterilization of the root canal can be achieved in a majority of all cases by judicious root canal instrumentation and irrigation. Thorough debridement and effective mechanical cleansing and shaping of the root canal in the presence of a mild irrigating solution will effect a sterilization far more predictable than can be achieved by use of drugs alone, according to Schilder (1967). Since parachlorophenol and cresatin have continued to be the most commonly used intracanal drugs, one must depend upon dilution of traces of these medications in the culture medium to minimize their in vitro antimicrobial effect.

## II. Bacteriologic Studies in Endodontics

Microorganisms were considered a probable etiological factor in pulp pathology as early as 1884, when Miller observed them in diseased pulp tissue. Dental literature credits a New York dentist by the name of Onderdonk of proposing in 1901 a culture test prior to filling the root canal. Later, La Roche and Coolidge stressed the bacteriologic sampling of teeth (1919). In 1927, Appleton confirmed the necessity of culturing root canals before filling them. Sommer and Crowley (1940), and Morse and Yates (1942), later demonstrated the need for strict asepsis during endodontic procedures, a method which is now advocated universally.

The most extensive studies on morphological forms seen in root canals were those of Crawford and Shanckle (1961); Mazarella, Hedman, and Brown (1955); Brown and Rudolph (1957); McDonald, Hare, and Wood (1957); and Winkler and Van Amerongen (1959). Most of these reports concur in the frequency with which some of the basic types appear, but they differ in the incidence of those types which appear less frequently.

The question of whether anaerobic bacteria are present is often posed, as the root canal would appear to be an ideal habitat for them. Investigations into the problem have resulted in conflicting reports. Several workers including Slack (1953) and McDonald, Hare and Wood (1957), found strict anaerobes to be present, while others like Morse and Yates (1941) and Shay (1947), disagreed with these findings.

In 1958 Leavitt, Naidorf, and Shugaevsky reported that anaerobes were disclosed in 33% of infected root canals. Shovelton and Sidaway (1960) found the frequency of obligate anaerobes in their study to be of the same order as that found by Cran (1956) and Brown and Rudolph (1957). In an investigation by Hobson in 1959, she concluded that strict anaerobes did not appear to be present in root canals which were studied in her series. In a recent study in 1969 by Goldman and Pearson, in which teeth were sampled after debridement of the root canals, the number of anaerobes isolated was

much smaller than reported in other studies where sampling was done prior to the canal debridement.

### III. Actinomyces israelii

The actinomycetes are a heterogeneous group of filamentous micro-organisms which are clearly related to "true bacteria," while superficially resembling fungi. The characteristic growth is a branched mycelium which tends to fragment into bacteria-like pieces. Some actinomycetes are acid fast. Many are free-living, particularly in the soil. The anaerobic species Actinomyces israelii, and some of the aerobic Nocardia and Streptomyces species produce disease in man and animals.

In culture, Actinomyces israelii is a Gram positive, non acid fast, filamentous organism which shows characteristic branching. The filaments break easily into short bacillary fragments with observable branching in the form of "V" or "X." In tissues, "sulfur granules" are formed which consist of a central mass of filamentous mycelia. A peripheral array of swollen eosin-staining "clubs" may be present.

For many years it was believed that Actinomyces bovis (Harz, 1877), isolated from lumpy jaw of cattle and Actinomyces israelii (Krusse, 1896); (Breed, Murray, and Smith, 1957) isolated from human actinomycosis, were



one species. However, studies by Erikson (1940, 1949); Thompson (1950) and Pine, Howell, and Watson (1960) provided morphological and biochemical evidence that there are two agents of actinomycosis: Actinomyces bovis, usually associated with bovine infection, and Actinomyces israelii, the common cause of human infections. Recently, Buchanan and Pine (1962) described a third agent of actinomycosis: Actinomyces proprionicus. This organism was isolated from human lacrimal canaliculitis.

Bergey (1907) is generally credited with the first isolation of actinomycetes from the normal mouths of humans. Since then there have been reports of the recovery from normal oral cavities of humans of organisms which appear to have been identical, or nearly so, with strains isolated from actinomycotic lesions in man. (Emmons, 1935, 1936 and 1938); (Ennever, et. al., 1951); (Morris, 1954).

In addition, there have been numerous reports of the isolation from the oral cavity of humans of one or more strains of organisms designated as Actinomyces. However, the data given in many of these reports were inadequate for the determination of the identity of the organisms described. (Bartels, 1952); (Crowley, 1941); (Ennever and Warner, 1952) (Garrod, 1952); (Villa, 1957). Thompson and Lovstedt (1951) isolated organisms from the normal human mouth which differed significantly from those isolated from lesions of actinomycosis and suggested that these strains be

designated as Actinomyces naeslundii. Garrod (1952) supported these findings. In 1959, Howell, Murphy, Paul, and Stephan compared 200 strains of Actinomyces isolated from the oral cavity in the absence of actinomycosis and 11 isolated from actinomycotic lesions. They found that the oral strains were of two main types, one corresponding to the organisms described by Thompson and Lovestedt (1951) and later Garrod (1952) for which the name Actinomyces naeslundii was proposed, and the other essentially identical to those isolated from lesions, which should be designated as Actinomyces israelii.

#### IV. Actinomycosis

Actinomycosis is a noncontagious, usually slowly developing, suppurative infectious disease affecting man and animals. Actinomyces israelii, a Gram positive, non acid fast, filamentous, anaerobic organism with a natural habitat in the human oral cavity, is the principal causative organism in human actinomycosis. Actinomyces bovis, a closely related organism which produces the disease in animals, was not found in the human oral cavity in a careful study in 1959 by Howell and co-workers. Nocardia asteroides, quite similar morphologically to Actinomyces israelii and Actinomyces bovis, but different bacteriologically in that it is aerobic and

partially acid fast, may produce lesions clinically similar to those of actinomycosis.

Because of the relative infrequency with which actinomycosis occurs, considering that the organisms are a part of the normal oral flora, the manner in which the disease is produced has been a controversial subject. The once prevalent concept that the organism gained entry via straw or other vegetable matter has been abandoned because:

- (1) The organism is a normal oral inhabitant.
- (2) Its prevalence is no greater in rural than in urban areas.
- (3) The strains of actinomycetes found on vegetable matter are considered incapable of producing human disease.

Trauma, incurred either accidentally or in the course of dental instrumentation, is presumed to afford the organism a portal of entry into the tissues. The organism itself is of low virulence, however, and factors other than mere entry into the tissues are thought to be necessary to establish infection. There is also evidence that actinomycosis may be an immunologic phenomenon, with repeated infections being required to produce active disease.

It is believed by some that pyogenic infections, commonly seen concurrently with actinomycosis, are important in that the other organisms

may improve growth conditions for the actinomycete. It should be pointed out that actinomycetes are cultured optimally at a slightly alkaline pH (7.4) whereas inflamed and infected or necrotic tissues are more likely to be slightly acid (Robbins, 1962); (Miller and Drake, 1951).

Actinomycosis is of worldwide distribution. It may occur at any age and is more common in males. Cervicofacial involvement is most common, with thoracic and abdominal involvement being presumed to be due to aspiration and swallowing of organisms from the oral cavity (Robbins, 1962). Classic symptoms include a relatively nontender, slowly enlarging reddish purple swelling which tends to point and to form one or more sinus tracts with drainage of a thin fluid containing brownish yellow "sulfur granules," (Monteleone, 1963).

Clinically, in its early stages, the disease may be mistaken for a dental abscess. Actinomycosis may occur as a central lesion in the jaws, and when it does, bony destruction may be present for some time before symptoms appear. The mandible is the more common site of oral involvement, with maxillary lesions being rare, (Owen and Macansh, 1965). Roentgenographically, when the jaws are involved, it is not unusual to find a periapical radiolucency suggestive of a dental granuloma (Thoma and Goldman, 1960). Diagnosis depends on identification of the organism, either in the "sulfur granules," in tissue section or by culture. The "sulfur granules"

may be crushed on a slide and observed microscopically for characteristic form. A tissue section may show the characteristic ray fungus surrounded by vast numbers of acute inflammatory cells, or the organism may be cultured under anaerobic conditions. Currently, therapy consists of massive doses of Penicillin for prolonged periods.

## CHAPTER III

### METHODS AND MATERIALS

#### I. Selection of the media

In this study, the following media were used and prepared to the manufacturer's directions:

- (a) Trypticase soy broth with 0.1% agar
- (b) Brain heart infusion broth
- (c) Brain heart infusion broth with 0.1% agar
- (d) Fluid thioglycollate broth
- (e) Glucose ascites broth with 0.1% agar

These media are currently the most popular in endodontic culturing procedures. The complete formulae and mode of preparation are described on Tables I, II, and III. The glucose ascites medium was prepared by using BBL\* Brain heart infusion broth to which 0.2% glucose was added, then sterilized, and after allowing to cool to 45° F., sterile ascitic fluid was added in a 10% ratio using careful and strict aseptic technique.

All media were prepared fresh for each series of experiments and sterilized in an autoclave for 20 minutes at 121° C. and 15 lbs. per square inch

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\*Baltimore Biological Laboratories, Cockeysville, Maryland

pressure. Uninoculated tubes of each batch of media sterilized were incubated each time the experiment was done to check for sterility or contamination of media.

## II. Test microorganisms

The bacteria used in the experiment were:

- (1) Strain of Actinomyces israelii, American Type Culture Collection #12103.
- (2) Streptococcus faecalis - Microbiology Department, Loyola University School of Dentistry, Maywood, Illinois. This strain was recovered from an infected root canal in the Endodontic Clinic at the school.

## III. Laboratory equipment

The usual laboratory equipment was used during the experimental procedures. Bacterial inoculation was done using sterile pipettes of proper sizes and observing aseptic techniques. All materials and equipment which required sterilization were autoclaved at 121° C. and 15 pounds per square inch pressure for 20 minutes.

TABLE I

BRAIN HEART INFUSION\* (BHI)

Gm/1

Calf Brain, Infusion from.....	200.0
Beef Heart, Infusion from.....	250.0
Peptone.....	10.0
Sodium Chloride.....	5.0
Disodium Phosphate.....	2.5
Dextrose.....	2.0

Final pH 7.4<sub>+</sub>

Preparation:

Dissolve 37 grams of the dehydrated material in a liter of distilled water. Heat while stirring until solution is complete. The addition of one gram of agar to this formula will produce BHI broth with 0.1% agar. For pour plates, 15 grams of agar are added to the dehydrated material.

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\*Baltimore Biological Laboratories, Cockeysville, Maryland



TABLE II

TRYPTICASE SOY BROTH\* WITH 0.1% AGAR

Gm/1

Trypticase Peptone.....	17.0
Phytone Peptone.....	3.0
Sodium Chloride.....	5.0
Dipotassium Phosphate.....	2.5
Dextrose.....	2.5
Agar.....	1.0

Final pH 7.3<sub>+</sub>

Preparation:

Suspend 31 grams of powder in a liter of distilled water.  
Heat while stirring and boil for one minute prior to sterilizing.

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\*Baltimore Biological Laboratories, Cockeysville, Maryland

TABLE III

FLUID THIOGLYCOLLATE\*

Gm/l

Trypticase.....	15.000
Cystine.....	0.500
Dextrose (anhydrous).....	5.000
Yeast Extract.....	5.000
Sodium Chloride.....	2.500
Sodium Thioglycollate.....	0.500
Resazurin.....	0.001
Agar.....	0.750

Final pH 7.1<sub>+</sub> 0.1

Preparation:

Suspend 29.5 grams of powder in a liter of distilled water. Mix thoroughly until suspension is uniform. Heat with frequent agitation and boil for one or two minutes or until solution is complete.

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\*Baltimore Biological Laboratories, Cockeysville, Maryland

The anaerobic equipment consisted of a glass jar with an air tight lid, an electric vacuum pump, and a cylinder of a gaseous mixture\* of 95% nitrogen and 5% carbon dioxide. After cultures were placed in the jar and the lid secured tightly, the jar was flushed with the gas for one minute. If screw capped tubes were placed in the jar, the caps were fitted loose, to allow penetration of gas into the tubes. The electric vacuum pump was used to evacuate air from the jar to 25 pounds of vacuum. Flushing with gas was done and subsequent evacuation and flushing repeated four or five times, leaving at the end an atmosphere of 95% nitrogen and 5% CO<sub>2</sub> in the jar. An indicator\*\* was used each time the jar was opened and refilled with gas, and also observed daily for color changes when cultures were incubated for days at a time. The anaerobic indicator was white when anaerobic conditions existed and blue when oxidized.

Prior to the start of the experiment, the anaerobic conditions using the same techniques and equipment were tested by cultivating and maintaining a strain of Clostridium perfringes and a strain of Clostridium tetanii, both obligate anaerobes, for seven days, without loss of the organisms. At no time during the experiment were anaerobic conditions in the jar not suitable.

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\* National Cylinder Gas Division of Chemetron Corporation, Chicago, Ill.

\*\*Gas Pak Indicator - Baltimore Biological Laboratories, Cockeysville, Md.

#### IV. Procedure

##### A. Streptococcus faecalis

In order to be able to compare aerobic vs. anaerobic methods, a strain of Streptococcus faecalis was used. A 48 hour culture of the organism was placed with a pipette on the first tube and mixed thoroughly. From this tube one ml. of the suspension was pipetted out and placed on the second tube and mixed. This was repeated until dilutions ranging from  $10^{-1}$  to  $10^{-20}$  were obtained of the organism being tested.

All of the media being tested had been dispensed in test tubes in 10 ml. amounts, except in the case of Fluid Thioglycollate, which contained 15 ml. per tube. An inoculum of 0.5 ml. from each dilution was placed into each of the tubes of media, totaling 100 tubes of inoculated media or 20 tubes for each of the media tested.

Pour plates of the dilutions  $10^{-9}$  to  $10^{-15}$  of the organism were done in the following manner in order to obtain viable counts of bacteria: One ml. of the above mentioned dilutions was placed in separate sterile Petri dishes and cool but molten BHI agar poured over. The contents were mixed by rotation and allowed to harden. Three plates for each dilution were inoculated. Incubation was done aerobically at  $37^{\circ}\text{C}$ . The plates were observed and counted at 72 hours. The inoculated tubes of media were also incubated

aerobically at 37° C. and observed for growth at 24 and 72 hours. Colony morphology studies and counts were obtained using an instrument\* devised for such.

For viable counts, plates were selected which contained between 30 and 300 colonies. Viewing the plates against a lighted background, the colonies were counted with the electric counter. Since plates were poured in triplicate, an average was established by adding the total counts and dividing by three. By multiplying the number thus obtained times the dilution factor used for those particular plates, an estimation of the bacterial population of the stock culture was obtained. For example, if the  $1:10^{-10}$  plate showed 40 colonies as an average, then  $40 \times 10^{10}$  organisms per ml. was the bacterial population of the original specimen.

B. Actinomyces israelii

A similar procedure as that used for Streptococcus faecalis was used. In this case the stock culture was allowed to incubate, anaerobically, in a medium of Fluid Thioglycollate and 10% fetal calf serum for 10 days. All cultures used in the series dealing with Actinomyces israelii were treated in the same manner. Serial dilutions were done using sterile distilled water

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\* Darkfield Quebec Colony Counter, AO Instrument Co. , Quebec, Canada

blanks until dilutions ranging from  $10^{-1}$  to  $10^{-10}$  were obtained. The inoculum consisted of 0.1 ml. of each of the dilution series. Tubes containing media were inoculated in duplicate, one series for aerobic incubation, and the other for obtaining viable colony counts was done by pipetting 0.1 ml. of each of the dilution series into a sterile Petri dish with blood agar and using a sterile glass rod to seed the inoculum on top of the medium. These were incubated anaerobically only, and done in triplicate for each dilution. The experiment dealing with Actinomyces israelii was repeated three times in the same manner. The incubated dilution series, both aerobic and anaerobic, were observed for results after 24, 48, and 72 hours and at the end of 10 days. Colony morphology studies were done using same techniques as previously described.

Checks were made during the duration of the experiment by Gram staining and microscopic examination to make certain of the identity of the organisms and to rule out contamination.

## CHAPTER IV

## RESULTS

I. Streptococcus faecalis

All media tested in this study were found to be adequate for the detection and cultivation of Streptococcus faecalis. At the 48 hour observation period, typical cloudiness of culture media in which the organism grew was noticed. The lowest concentration of inoculum initiating growth was the same for all media. This was the inoculum extracted from the tube containing the  $10^{-11}$  dilution. At the 72 hour observation period the results had not changed. Dilutions of the organism of more than  $10^{-11}$  caused it to be diluted out, thus inocula from the tubes  $10^{-12}$  and over carried no viable cells and did not start growth in any media. It was calculated that  $284 \times 10^{10}$  organisms per ml. existed in the original specimen from which the dilutions were made. The Streptococcus faecalis colonies in the BHI agar plate were quite small, circular, convex, entire, smooth, and creamy in color and texture. Growth in the broth media was of uniform turbidity with some sedimentation. In the semisolid media turbidity was uniform and there was less sedimentation or none at all. Microscopic examination of samples from the media and from the pour plates showed Gram positive cocci in small chains.

## II. Actinomyces israelii - aerobic incubation

The aerobic cultivation method showed no discernible colonial formation or turbidity in any of the media tested at the 48 hour observation period. When observed at 72 hours, colonies in the Glucose Ascites broth with 0.1% agar and in the Fluid Thioglycollate media which had been inoculated with organisms from tubes of the higher concentrations were visible. The results are summarized in Table number IV.

At the end of the 10th day it was evident that BHI broth did not support the growth of the organism. The lowest concentration of inoculum showing growth was  $10^{-4}$  in the Fluid Thioglycollate medium. Glucose Ascites, with 0.1% agar was able to exhibit growth when inoculated from the  $10^{-3}$  dilution. BHI broth with 0.1% agar required a larger inoculum to initiate growth.

Those tubes of media in which growth was apparent at the 72 hour observation period contained a larger number of colonies than they did at the 48 hour period. The level at which the colonies grew was well below the free surface of the media. The size of the colonies did not differ greatly from one medium to the other.

## III. Actinomyces israelii - anaerobic incubation

As shown on Table V, using anaerobic incubation, Fluid Thioglycollate and Glucose Ascites with 0.1% agar showed colony formation in 48 hours in those tubes which were inoculated from the  $10^{-1}$  dilution. At 72 hours, tubes



TABLE IV

Actinomyces israelii : Aerobic Cultivation

LOWEST CONCENTRATION OF INOCULUM SHOWING GROWTH

Observation Period	BHI BROTH			BHI BROTH AND 0.1% AGAR			TRIPTICASE SOY AND 0.1% AGAR			GLUCOSE ASCITES AND 0.1% AGAR			FLUID THIO-GLYCOLLATE		
	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
	NG	NG	NG	NG	NG	NG	NG	NG	NG	10 <sup>-1</sup>	10 <sup>-1</sup>	10 <sup>-1</sup>	10 <sup>-1</sup>	10 <sup>-1</sup>	10 <sup>-1</sup>
	NG	NG	NG	10 <sup>-2</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-3</sup>	10 <sup>-3</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-4</sup>
48 hours															
72 hours															
10 days															

LEGEND: NG - NO GROWTH

TABLE V

Actinomyces israelii : Anaerobic Cultivation

## LOWEST CONCENTRATION OF INOCULUM SHOWING GROWTH

Observation Period	BHI BROTH			BHI BROTH AND 0.1% AGAR			TRIPTICASE SOY AND 0.1% AGAR			GLUCOSE ASCITES AND 0.1% AGAR			FLUID THIO-GLYCOLLATE		
48 hours	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	10 <sup>-1</sup>	NG	10 <sup>-1</sup>	10 <sup>-1</sup>
72 hours	NG	NG	NG	10 <sup>-1</sup>	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-2</sup>	10 <sup>-2</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-3</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>
10 days	10 <sup>-3</sup>	10 <sup>-2</sup>	10 <sup>-2</sup>	10 <sup>-4</sup>	10 <sup>-4</sup>	10 <sup>-4</sup>	10 <sup>-4</sup>	10 <sup>-4</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-5</sup>	10 <sup>-5</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-5</sup>

LEGEND: NG - NO GROWTH

TABLE VI

Actinomyces israelii, anaerobic incubation:

A. Viable counts.....225 colonies, on  
plate seeded with  $10^{-4}$  dilution.

B. Stock culture contained  $2.25 \times 10^{-6}$  organisms per ml.

C. Inoculum used was 0.1 ml. from each tube.

<u>DILUTION</u>	<u>NO. OF ORGANISMS TRANSFERRED</u>
$10^{-1}$ (1:10) .....	22,500.00
$10^{-2}$ (1:100) .....	2,250.00
$10^{-3}$ (1:1,000) .....	225.00
$10^{-4}$ (1:10,000) .....	22.50
$10^{-5}$ (1:100,000) .....	2.25

containing Trypticase Soy with 0.1% agar and BHI with 0.1% agar started to show growth. The BHI broth tubes were clear.

At the 10 day observation period it was found that the lowest concentration of inoculum producing growth was from the  $10^{-5}$  dilution. The most sensitive media were Fluid Thioglycollate and Glucose Ascites broth with 0.1% agar, followed by BHI with 0.1% agar. The BHI broth showed the presence of a heavy, flocculent sediment at the bottom of the tubes inoculated by the  $10^{-2}$  dilution. The other media showed colony formation, which were uniformly distributed throughout the contents. These colonies were discrete, variably sized, lobulated, cauliflower like, and creamy white in color. The colonies grown under anaerobic conditions were much larger in size than the colonies seen when incubation was done otherwise.

Gram stains were made from samples recovered from the different media. Under the microscope, Gram positive, filamentous organisms were observed, and occasionally rod forms. The filaments were slender and of variable thickness with either straight or tapered ends. They stained heavily. The appearance of rod forms was due to the breaking up of filaments during preparation of the slides. The number of organisms in the stock culture was  $225 \times 10^4$  per ml. The appearance of the colonies on the blood plates was that all were quite similar, white, larger than 0.5 mm., but not

larger than 2 mm. , raised to hemispherical forms with a finely lobate surface and a flat filamentous edge. When examined by transmitted light they were creamy white. Their consistency in general was soft but granular in nature and not easily emulsified when smears were prepared.

During the experimental procedure close supervision was maintained. Microscopic examination of Gram stained smears frequently prepared from the different colonies failed to show anything but filamentous Gram positive forms. The anaerobic conditions in the jar were maintained at all times, as shown by the indicators used.

## CHAPTER V

## DISCUSSION

The nutritive requirements of microorganisms vary greatly; some will grow readily in synthetic media containing inorganic salts, including an ammonium salt, together with a simple organic compound such as glucose or asparagin as a source of carbon and energy. In general, microbiological media have been developed on a trial and error basis about a basic nutrient medium containing peptone and the water soluble material, largely extractions from animal tissues such as muscle, liver, brains, and the like. The basal nutrient solution may be modified in a variety of ways. It can be solidified to various degrees by the addition of agar in different concentrations, or enriched with serum, ascitic fluid, defibrinated blood and similar substances, to support the growth of more demanding or fastidious bacteria. Nutritional requirements apparently depend largely on the enzyme systems possessed by given microorganisms. These in turn determine their ability or lack of ability to synthesize needed amino acids, vitamins, or other growth factors.

Although man and animals in general perish when deprived of atmospheric oxygen, microorganisms range from those growing only in the presence of atmospheric oxygen to those which will not grow in its presence.

They have been divided into aerobes, anaerobes, facultative organisms, and microaerophilic organisms on the basis of their oxygen requirements.

Aerobes are those microorganisms requiring oxygen in order to grow. Those which will grow only in the presence of air are often termed obligate aerobes.

Microorganisms which are classified as anaerobes live only in the absence of oxygen. Obligate anaerobes are apparently unable to grow when oxygen is present. Why oxygen is so inimical to the growth of anaerobes is obscure, but it is believed that in the presence of oxygen, hydrogen peroxide is produced which accumulates in toxic concentrations because the organism lacks the enzyme catalase which is necessary to break down this substance as fast as it is formed. Most aerobes produce catalase, whereas most anaerobes function only when in a reduced state. Actinomyces israelii has been classified as an obligate anaerobe in many texts, including Bergey's Manual of Determinative Bacteriology.

Many of the microorganisms in our environment are neither strict aerobes nor anaerobes, but are able to live in either the presence or the absence of air. These are spoken of as facultative microorganisms. Streptococcus faecalis is a facultative organism and this is evidenced by the fact that in this experiment it grew just as well at the top of tubes which contained various concentrations of oxygen throughout as at the bottom.

Microaerophilic organisms are those which require a reduced oxygen supply to survive. Since in this study this type of organism does not play a role, they will not be discussed.

The results obtained in the Streptococcus faecalis experiments showed that relatively few organisms (less than three) of the strain used were needed to initiate growth in all of the culture media tested in this study. These results are comparable with Grossman's results in a study in which he concluded that generally a small number of organisms of the type frequently found in root canals are necessary to start growth in culture media commonly used in endodontic practice. Grossman used in his study (1966) trypticase soy broth with agar, cooked meat broth, and thioglycollate. In our study the cooked meat was substituted for by BHI broth\* with 0.1% agar added, and results were similar for all. The addition of agar to some of the media did not alter the sensitivity of the media for the detection of Streptococcus faecalis. This organism grew well throughout the tubes, since its oxygen requirements are not critical and it can live either in its presence or absence.

It has been well established that the most predominant group of organisms invading the root canals are the streptococci. Of these, the hardest

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to eliminate is Streptococcus faecalis. All the media tested here are capable of detecting its presence in 48 hours using commonly accepted bacteriologic procedures in root canal treatment, even when present in very small concentrations.

The relationship of cervicofacial actinomycosis to dental sepsis has been known for some time. Cope (1915) noted the possible connection and Sanford and Nagath (1922), found that "Immediately following extraction of a tooth, the lesion appears." Stones (1962) pointed out that after extractions, actinomycosis should be suspected when there is a delay in healing or a recurrence of induration without an obvious explanation.

In 1965 Mitchell corroborated that there is little doubt that tooth extraction or mandibular fracture involving a tooth socket, can be a starting point for actinomycosis. He studied 46 lesions from which Actinomyces were obtained. Thirty-six of these lesions were regarded as examples of cervicofacial actinomycosis. Ten lesions from which Actinomyces were similarly obtained were grouped as acute or subacute dental abscesses. A relationship thus was established between actinomycosis and the dental abscess. Earlier, in 1963, Killey reported on eleven patients, differentiating between painful and painless lesions. The majority of these lesions (six) had been acutely painful, and clinically undistinguishable from acute

alveolar abscesses. He noted that there appeared to be two types of infections, one directly related to actinomycosis and eradicated only after protracted antibiotic therapy, and the other type which clears up easily with minimal treatment. The infrequent occurrence of actinomycotic lesions in the oral cavity in comparison to the innumerable opportunities arising each year from the thousands of extractions, root canal and periodontal procedures leads one to appreciate the resistance of the host or the low virulence of the organism. Animal inoculation has not completely demonstrated the relative pathogenicity of the actinomyces, for no animal is available that is consistently susceptible to them, and in which progressive disease can be induced.

Different investigators have reported on the difficulty in maintaining cultures of Actinomyces israelii. Aerobic incubation in BHI Broth\* is of no value for the detection of this organism. This is clearly due to the fact that the oxygen concentration in this liquid medium is too high for this anaerobe, and it will not survive. If the incubation is done under strict anaerobic conditions, the organism will grow, although an inoculum of a large number of organisms is required, and incubation for periods much longer than 72 hours needed. Howell and co-workers showed in 1959 that

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aerobic cultivation and growth of Actinomyces israelii was dependent on both the size of the inoculum and the medium employed. BHI Broth is not a suitable medium for the detection of Actinomyces israelii, even when anaerobic conditions are used for incubation. Organisms may exist in root canals in pure cultures or mixed. In the case of mixed infections, streptococci are likely to be one of the organisms present. It is easy for streptococci to overgrow Actinomyces israelii in BHI Broth, and its presence may never be detected even though it may have existed in the root canal in concentrations large enough to initiate growth in other media or if found in pure culture.

Our study showed that the addition of 0.1% agar to the BHI Broth improved chances for the detection of Actinomyces israelii. When incubated aerobically, it took 10 days and a large inoculum to show growth. Anaerobic incubation showed growth in 72 hours with a large inoculum. At the end of ten days those tubes inoculated with dilutions of  $10^{-4}$  showed growth also. The amount of growth in the aerobic cultivation was well below the free surface of the medium, supporting the fact that agar permits varying levels of oxygen tension and anaerobes may grow toward the bottom of the tube. Under anaerobic conditions the colonies grew luxuriantly and at all levels of the media. This medium did not prove very adequate for detection of Actinomyces israelii, and in an endodontic practice it would require an

inoculum of at least 225 organisms to show growth when incubating aerobically for ten days. This is hardly practical in an endodontic practice.

Trypticase Soy Broth\* with 0.1% agar is a general purpose semisolid medium, which contains two peptones, Trypticase and Phytone. It supports the growth of a wide variety of organisms. Many bacteriologists have used it for growing anaerobes such as Bacteroides and the pathogenic Clostridia, either in deep cultures or a surface cultures, with higher concentrations of agar incubated in anaerobic jars. This medium proved to be more sensitive to the presence of Actinomyces israelii than the preceding. It took a smaller inoculum to start growth anaerobically than aerobically. This medium has shown to have excellent growth promoting properties for fastidious organisms. Leavitt and co-workers found that the incidence of Actinomyces present in root canals increased when this medium was used by them (1955) on a two year clinical study. In our study, aerobic cultivation of 72 hours or less was not sufficient for the detection of growth of these organisms. Under anaerobic conditions it took an inoculum of at least 2,250 organisms to start growth at 72 hours. Again, colony size and level of growth in the media were different between those tubes incubated aerobically and anaerobically, and this is attributed to the anaerobic properties of the organism being tested.

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Glucose Ascites\* with 0.1% agar and Fluid Thioglycollate\* were the only media capable of showing good growth of Actinomyces israelii at 72 hours on both methods of incubation. Crowley (1944) had been able to identify Actinomyces israelii from the root canal recovered by the use of glucose ascites medium. She advocated anaerobic incubation for four to six days at 37 C. for the recovery of Actinomyces israelii from infected teeth. The addition of 0.1% agar makes the medium more suitable for the recovery of Actinomyces israelii when incubated aerobically for 72 hours if the inoculum is at least 22,500 organisms as shown in our study.

Thioglycollate medium was designed by Brewer (1940) for rapid cultivation of anaerobes as well as aerobes. Incorporation of caseine peptone was introduced by Vera in 1944. It is capable of supporting good growth of a great variety of fastidious organisms. Thioglycollic acid or its sodium salt are reducing substances. The addition of a reducing substance to the medium maintains anaerobic conditions which exist for a time after heat sterilization. Anaerobiosis is also assisted by the glucose and by the small amount of agar which prevents convection currents in the medium. This medium is stored at room temperature since it absorbs more oxygen at lower temperatures. The addition of resazurin acts as a reduction potential indicator. This medium has been recommended by many for use as a general

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utility medium and for examination of blood cultures and all other materials in which the presence of a variety of aerobic, anaerobic, or facultative organisms is possible. In root canal culture work, the medium is more productive than the infusion media which were formerly popular. Our study showed that this medium was more sensitive than the others tested for the detection and cultivation of Actinomyces israelii, and it generally required smaller concentrations of organisms as inoculum to show growth.

During the course of this study an attempt was made to recover and isolate Actinomyces israelii from root canals of teeth being treated at the endodontic clinic at Loyola University School of Dentistry, Maywood, Illinois. Fluid Thioglycollate Broth\* was employed as the medium and inoculated tubes were incubated "aerobically" for 72 hours. A total of 46 positive cultures were subcultured in duplicate in blood agar slants and these were incubated aerobically and anaerobically for four days. Using routine bacteriologic procedures, no Actinomyces israelii were recovered from these cases. Since the bacteriologic sampling was done on these cases after debridement of the root canals, and since this study has shown that it takes an inoculum of at least 22,500 organisms to start growth in thioglycollate medium when incubated "aerobically," it is assumed that the concentration of the organisms, if present, had been diluted below that required to start growth in this medium.

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The relationship of Actinomyces israeli to the dento-alveolar abscess has been discussed in this paper. The fact that this organism may invade the root canal has been established by its recovery from such by investigators mentioned in this paper. That the root canal may serve as a portal of entry for the organisms to the periapical tissues is a logical conclusion, it all being dependent on host-parasite relationship. Whether or not the organism can be associated with "flare-ups" of teeth properly treated by endodontic procedures has never been established, nor has it been established that patients have developed cervicofacial actinomycosis subsequent to endodontic treatment. At any rate it appears that properly executed procedures of root canal debridement and obliteration are not as a general rule associated with clinical cervicofacial actinomycosis. Media presently used in endodontic procedures are not sensitive enough to detect the presence of Actinomyces israelii in the root canals if they exist there in small numbers and even more so if aerobic incubation of samples for short periods is employed. Anaerobic incubation utilizing Fluid Thioglycollate Broth is the most sensitive method for detection of Actinomyces israelii in the practice of endodontics. The other media tested required longer periods of incubation and larger concentrations of organisms to show growth.

## CHAPTER VI

## SUMMARY

Actinomyces israelii is the principal causative agent of cervicofacial actinomycosis and a very close relationship exists between the dental abscess and actinomycosis. Five culture media which are most commonly used in endodontics were tested and compared as to their ability to detect the presence of Actinomyces israelii using different concentrations of organisms as the inoculum. Tests were done three times and aerobic and anaerobic methods of cultivation were used. Results were observed and recorded at intervals of 48 hours, 72 hours, and 10 days. Included in the study was a similar experiment using Streptococcus faecalis, which is known to be the most difficult organism to eradicate from infected root canals. The results showed that the media tested were indeed adequate for the detection of Streptococcus faecalis under normal conditions, but not adequate for the detection of Actinomyces israelii unless strict anaerobic conditions for incubation are observed or unless cultivated for periods of more than 72 hours.



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PLATE I



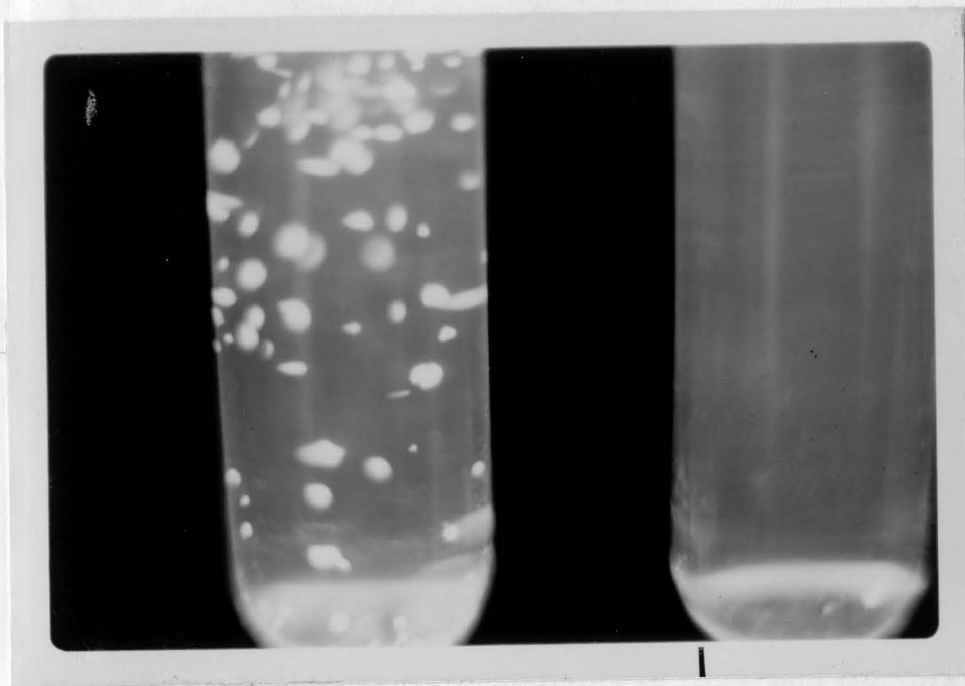
ANAEROBIC EQUIPMENT

Left to Right: Vacuum pump, anaerobic jar, and gas cylinder.

Right: Glucose series medium after 48 hours incubation, incubated aerobically



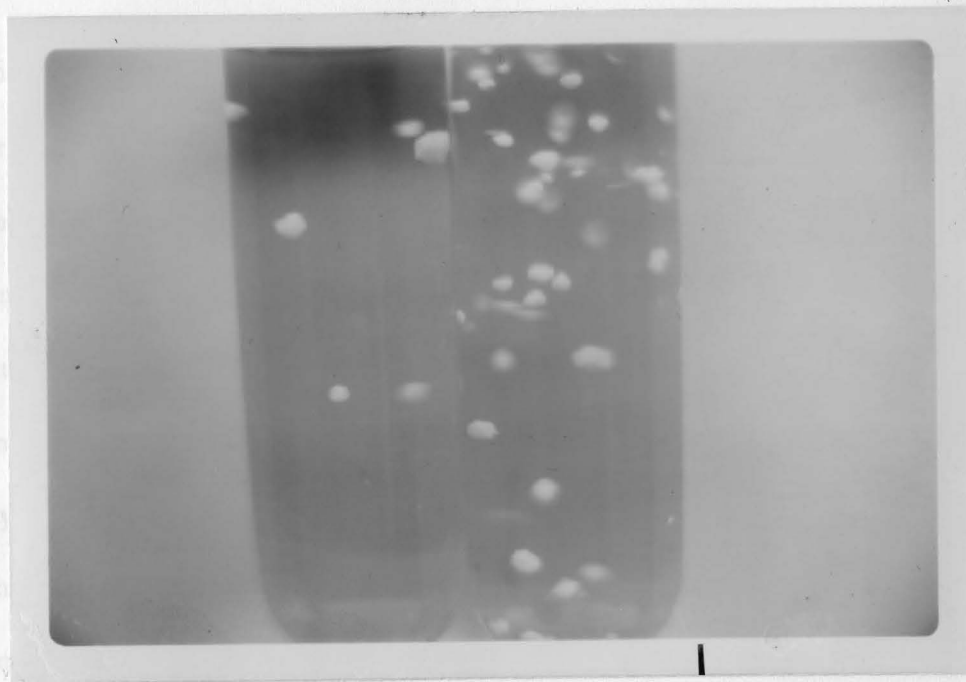
PLATE II



Left: *Actinomyces israelii* colonies incubated anaerobically  
in Glucose Ascites with 0.1% agar after 10 days

Right: Glucose ascites medium after 48 hours inoculation,  
incubated aerobically

PLATE III



Left: *Actinomyces israelii* colonies in fluid Thio glycollate medium, anaerobic cultivation after 72 hours.

Right: Colonies in Fluid Thioglycollate medium, anaerobic cultivation after 10 days.

5-19

Date

Madame  
Signature of A. H. L.

## APPROVAL SHEET

The thesis submitted by Dr. Orlando Muñoz-Noya has been read and approved by three members of the Graduate School Faculty.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with references to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

5-19-70

Date

John V. Madonia D.D., Ph.D.

Signature of Advisor